Kent Academic Repository
Full text document (pdf)

Citation for published version

McCallie, Blair R. and Parks, Jason C. and Griffin, Darren K. and Schoolcraft, William B. and Katz-Jaffe, Mandy G. (2017) Infertility diagnosis has a significant impact on the transcriptome of developing blastocysts. Molecular Human Reproduction, 23 (8). pp. 549-556. ISSN 1360-9947.

DOI

https://doi.org/10.1093/molehr/gax034

Link to record in KAR

http://kar.kent.ac.uk/62408/

Document Version

Author's Accepted Manuscript

1 **Infertility Diagnosis has a Significant Impact on the Developing Blastocyst's**

2 **Transcriptome**

3

4 **Running title:** Infertility diagnosis impacts blastocyst transcriptome

5

6 **Authors:** Blair R McCallie*[+1, 2]
7 Jason C Parks[+1, 2]
8 Darren K Griffin[2]
9 William B Schoolcraft[3]
10 Mandy G Katz-Jaffe[1, 3]
11

12 [1] Fertility Labs of Colorado, Lone Tree, CO, USA

13 [2] University of Kent, Canterbury UK

14 [3] Colorado Center for Reproductive Medicine, Lone Tree, CO, USA

15 *Corresponding author

16 [+]These authors contributed equally to this work

17

18

19

20

21

22

23

24

25

26

27

28

29 **Abstract**

30 Infertility is a disease that affects 1 out of 6 reproductive-age couples. The causes are diverse

31 including, but not limited to, polycystic ovaries (PCO), male factor (MF), and when all sources

32 have been ruled out, the couple is defined as having unexplained infertility. While each etiology

33 is distinct, they are all typically associated with lower implantation potential and poorer

34 pregnancy outcomes. The aim of this study was to characterize the global transcriptome of

35 human blastocysts, from patients presenting with specific infertility etiologies, to elucidate novel

36 biological pathways that may influence downstream implantation. Surplus, cryopreserved, day

37 5 blastocysts of transferrable quality were donated with Institutional Review Board (IRB)

38 approval and patient consent for transcriptome microarray analysis. The human blastocyst

39 transcriptome contained 13,136 annotated genes with the most significant alteration observed

40 for blastocysts derived from infertile PCO patients. 869 genes were differentially expressed in

41 PCO blastocysts, 348 in MF blastocysts, and 473 in blastocysts from unexplained etiology

42 compared to fertile, donor controls (P<0.05; >2-fold). Validation utilizing real-time quantitative

43 PCR was performed on genes belonging to enriched pathways including: BCL2L10, HSPA1A,

44 HSPA1B, ATF3, FGF9, LEFTY1, LEFTY2, GDF15, INHBA, AJAP1, CDH9, and LAMA4

45 (P<0.05; >2-fold). Functional annotation of biological and molecular processes revealed both

46 similarities, as well as differences, across the infertility groups. All etiologies displayed

47 transcriptome alterations in signal transducer activity, receptor binding, reproduction, cell

48 adhesion, and response to stimulus. However, blastocysts from PCO patients were also

49 enriched for apoptosis while MF blastocysts displayed enrichment for cancer processes.

50 Blastocysts from couples with unexplained infertility were enriched for pathways related to

51 various disease states which included mTOR and adipocytokine signaling. In conclusion,

52 underlying patient infertility diagnosis is reflected in the blastocyst transcriptome, which may

53 then impact developmental competence and implantation outcomes. Ongoing research could

54   result in the development of new laboratory or clinical therapies, improving patient diagnosis

55   and management.

56

57   **Keywords:** polycystic ovaries, male factor, unexplained Infertility, transcriptome, gene

58   expression

59

60

61

62

63

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

Introduction

The World Health Organization (WHO) estimates that 1 out of 6 couples struggle with infertility and the origins are equally distributed between male and female. There are many different causes of infertility including, among others, polycystic ovaries (PCO) and male factor (MF). Infertility can be the result of a variety of problems ranging from genetic to hormonal and even environmental. When all known sources have been ruled out, the couple is defined as idiopathic or unexplained.

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and a major cause of female factor infertility (Sirmans and Pate, 2013). It is the result of hormonal imbalances, typically excess androgen production, which lead to rare or irregular ovulation (Krishnan and Muthusami, 2017). Unlike PCOS, women diagnosed with polycystic ovaries (PCO) do not have a metabolic condition but have ovaries with abnormally high follicle counts and can still possess hormonal imbalances. PCO is far more common than PCOS, affecting anywhere from 20-30% of the population and the causes are largely unknown (Koivunen et al. , 1999). PCO patients are often infertile due to anovulation, thereby requiring assisted reproductive technologies (ART) to conceive. High miscarriage rates are associated with this infertility phenotype, as well as decreased fertilization after IVF, suggesting poorer quality oocytes and embryos (Hardy et al. , 1995).

Male factor infertility, which is almost always defined as abnormal semen analysis based on WHO guidelines, is solely responsible for 20-30% of human infertility and is a contributing factor in half of all couples presenting for ART (Agarwal et al. , 2015). Problems with sperm production can originate from many different factors including hormonal, environmental, and even on a physical level within the testicle, causing problems with the seminiferous tubules. These tubules contain the Sertoli cells that act as nourishment for developing germ cells and are the location for spermatogenesis. Poor semen parameters have been shown to result in

105   delayed and failed fertilization, as well as compromised embryo development and quality (Janny

106   and Menezo, 1994, Ron-el et al. , 1991).

107   Unexplained infertility is diagnosed in about 15-30% of infertile couples and is difficult to

108   treat due to the unknown underlying etiology (Practice Committee of the American Society for

109   Reproductive, 2006). It is defined as the inability to conceive after 12 months of regular,

110   unprotected intercourse and when all recommended fertility assessments fail to reveal any

111   anomaly (Quaas and Dokras, 2008). Patients can present with varying infertility histories

112   including multiple IVF failures, poor embryo development, as well as lengthy periods of infertility.

113   A retrospective review of 45 studies found that couples with this diagnosis have, on average, a

114   1-4% chance of achieving pregnancy during any given menstrual cycle without utilizing ART

115   (Guzick et al. , 1998). Nevertheless, 40-60% will spontaneously conceive within 3 years

116   (depending on the female partner's age) and this rate can increase to as high as 75% with the

117   use of ART (Daniela Galliano, 2015). ART techniques can also potentially help further address

118   the cause of infertility in these patients (i.e. low fertilization rates, embryo fragmentation,

119   abnormal oocytes, etc.) as well as improve time to conception.

120   A fertilized oocyte must not only facilitate the syngamy of the male and female genomic

121   contributions but also undergo a series of cellular divisions before embryonic genome activation

122   is initiated (Fragouli et al. , 2013). Both the timing of the activation, as well as the synchrony of

123   genes activated, must be accurately controlled to produce a blastocyst stage embryo that is

124   viable and developmentally competent for implantation to occur (Latham and Schultz, 2001). In

125   the mouse model, studies have observed two waves of embryonic gene transcription, the first

126   corresponding to zygotic genome activation which occurs at the 1-2 cell stage, and the second

127   occurring during the morula-to-blastocyst transition (Hamatani et al. , 2004a). While these

128   transcriptional events are similar in the human embryo, the timing is different with the zygotic

129   genome activation occurring at the 4-8 cell stage (Niakan et al. , 2012). Any irregularities during

130   this critical time can lead to embryos that are incompetent and unable to implant.

131   The interactions between the blastocyst and the uterus that result in successful

132 implantation are directed by an equally complex molecular dialogue (Fitzgerald et al. , 2008).

133 Uterine receptivity has been extensively studied on all molecular levels, including the cross-talk

134 between the embryo and endometrium which is quite extensive and results in an environment

135 ideal for embryo adhesion and placentation (Miravet-Valenciano et al. , 2015). It has also been

136 shown that viable mouse embryos have a specific gene expression profile that favors uterine

137 attachment and invasion of the maternal endometrium. Chaen et al. found that ovarian

138 estrogen indirectly coordinates mouse blastocyst adhesion through integrin activation in the

139 blastocyst (Chaen et al. , 2012). Additionally, a mammalian model for blastocyst activity has

140 shown that specific molecular signaling directs either blastocyst activation or dormancy,

141 affecting implantation competency (Hamatani et al. , 2004b). Our lab has previously reported

142 that differential mouse trophectoderm gene expression following embryo biopsy is associated

143 with murine blastocyst implantation success. Specifically, higher gene expression of B3gnt5,

144 Cdx2, Eomes, and Wnt3a were predictive of sustained implantation. In contrast, decreased

145 gene expression of Eomes and Wnt3a were associated with absorption or pregnancy loss and

146 decreased gene expression of B3gnt4 and Cdx2 were observed with negative outcomes (Parks

147 et al. , 2011).

148   There is limited knowledge of the human preimplantation embryo transcriptome and how

149 it correlates to pregnancy outcomes. Jones et al. examined the transcriptome of human

150 trophectoderm biopsies and identified more than 7000 transcripts expressed exclusively in

151 viable blastocysts (Jones et al. , 2008). A more recent study performed single-cell RNA

152 sequencing on both human and mouse preimplantation embryos to determine a dataset of

153 genes that are important for pluripotency (Blakeley et al. , 2015). Ongoing transcriptome

154 analysis in our lab revealed differential gene expression from blastocysts obtained from PCO

155 women compared with donor controls. Over 800 genes were found to be disrupted in these

156 PCO blastocysts in addition to 12 altered protein biomarkers, demonstrating a link between

157 patient infertility phenotype and embryo development (Katz-Jaffe et al. , 2010).

158   The objective of the present study was to further explore the global transcriptome of

159 human blastocysts from patients with differing infertility etiologies, specifically PCO, male factor,

160 and unexplained infertility, to uncover novel biological pathways associated with their infertility

161 that may influence downstream implantation outcomes. These findings will further our

162 understanding of the impact of infertility diagnoses on the embryonic molecular signature at the

163 time of implantation, and may lead to refined lab-based and clinical approaches for improving

164 IVF outcomes.

165

166 **Materials and Methods**

167 **Human blastocysts**

168   Surplus, cryopreserved, anonymous, human blastocysts from IVF patients with specific

169 infertility diagnoses were donated with Institutional Review Board (IRB) consent. All embryos

170 were considered to be transferable quality with a grade of 3BB or better on day 5 of embryo

171 development (Gardner and Schoolcraft, 1999). Either slow freezing or vitrification protocols

172 were used to cryopreserve the blastocysts (Kuwayama, 2007, Veeck et al. , 2004) which were

173 grouped according to a single distinct infertility diagnosis: n=50 young donor oocyte controls

174 with no male factor infertility; n=50 polycystic ovaries (PCO); n=50 male factor infertility (MF);

175 and n=50 unexplained infertility (UE). Every blastocyst used in this study came from a different

176 patient (female <38 years old, male <40 years old) and all patients had successful pregnancies

177 from the same IVF cohort as the blastocyst used for research. Patients diagnosed with PCO

178 had polycystic ovaries confirmed by ultrasound but did not have any endocrine or metabolic

179 abnormalities, as determined by androgen levels, fasting glucose and insulin levels, and oral

180 glucose tolerance testing. MF infertility patients were all diagnosed based on WHO guidelines

181 as oligoasthenoteratozoospermia with sperm concentration <15 million/ml, motility <32%, and

182 <4% normal morphology. UE infertility was defined following a negative fertility workup which

183 included normal semen analysis, normal ovarian reserve testing, and normal uterine

184 assessment with no prior failures or missed abortions.

185 **Blastocyst thaw and RNA isolation**

186 Blastocysts were either thawed or warmed using routine laboratory procedures, with an

187 overall 95% survival rate. Blastocysts in each distinct infertility diagnosis group were pooled

188 (n=25 per pool, 2 pools per group) and RNA was isolated using the PicoPure RNA Isolation Kit

189 (ThermoFisher Scientific, Grand Island NY) per the manufacturer's instructions with minor

190 modifications. Briefly, blastocysts were lysed in 10ul of Extraction Buffer before adding 1

191 volume of 70% ethanol and binding to a silica-based membrane. Samples were then washed

192 and on-column deoxyribonuclease treated (Qiagen, Valencia CA) prior to elution in 20ul and

193 storage at -80°C.

194 **Microarray hybridization**

195 Isolated RNA from each group was reverse transcribed, amplified, and labeled using the

196 LowInput QuickAmp Labeling Kit (Agilent Technologies, Santa Clara CA). Quantification and

197 quality of total RNA was performed using the High Sensitivity RNA ScreenTape on a 4200

198 TapeStation System (Agilent Technologies). Quantification and specific activity of labeled

199 cRNA was determined using the NanoDrop® ND-1000 spectrophotometer (ThermoFisher

200 Scientific). 600ng of cRNA was then applied to the SurePrint G3 Human Gene Expression

201 Microarray containing 50,599 biological features (Agilent Technologies) per the manufacturer's

202 instructions and hybridized in a rotating oven for 17 hours at 65°C. Arrays were washed and

203 then scanned using a DNA Microarray Scanner C (Agilent Technologies). Feature Extraction

204 software was utilized to extract gene expression data (Agilent Technologies).

205 **Real-time quantitative PCR Validation**

206 RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit

207 (ThermoFisher Scientific). cDNA was diluted 1:5 in nuclease-free water and Real-time

208 quantitative PCR (RT-qPCR) was performed for validation of specific differentially expressed

209 genes identified from the transcriptome analysis. Absolute expression was quantified relative to

210 a standard curve using slope and PCR efficiencies and normalized to a stable housekeeping

211 gene, GAPDH. Briefly, Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) was

212 combined with 5uM primer mix and 5ul diluted cDNA for a total volume of 25ul. The reaction

213 was incubated at 95℃ for 10 minutes, followed by 40 cycles of amplification at 95℃ f or 15

214 seconds and 60℃ for 1 minute with a final dissociation stage for melt curve analysis.

215 **Statistical analysis**

216   Transcript analysis was performed using GeneSpring software (version 7, Agilent

217 Technologies), including principal component analysis (PCA), unsupervised hierarchical

218 clustering, one way ANOVA and unpaired t-test with Benjamini-Hochberg correction

219 (significance at $P<0.05$). qPCR analysis was performed with REST 2009 software (Qiagen)

220 which uses the correction for exact PCR efficiencies with mean crossing point deviations

221 between sample and control groups to determine an expression ratio that is tested for

222 significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined

223 as $P<0.05$.

224 **Ethical approval**

225   All human participants were consented and this study was approved by an institutional

226 review board.

227

228 **Results**

229 **The blastocyst transcriptome according to infertility diagnosis**

230   The overall human blastocyst transcriptome contained 33,587 gene transcripts which

231 included numerous splicing variants and isoforms, revealing 13,136 annotated genes. PCA and

232 unsupervised hierarchical clustering distinguished each of the four blastocyst groups by their

233 transcriptomes (Figure I). The most significant transcriptome variation was observed in

234   blastocysts derived from infertile PCO patients. Compared to donor controls, significant

235   differences in transcription (>2 fold; $P<0.05$) were observed for 869 genes in PCO blastocysts,

236   348 genes in MF blastocysts, and 473 genes in blastocysts from couples with unexplained

237   infertility (Figure II). Both upregulation and downregulation were observed in each group: PCO

238   = 647 increased, 222 decreased; MF = 143 increased, 205 decreased; UE = 305 increased, 168

239   decreased (Table I).

240   Functional annotation of PCO blastocysts was performed using DAVID

241   (https://david.ncifcrf.gov) which revealed significant differences in gene ontology including: cell

242   communication, differentiation and adhesion, reproduction, transcription factor activity,

243   regulation of apoptosis, receptor binding, signal transducer activity, and response to hormone

244   stimulus. Pathway analysis identified enriched biological processes with altered transcripts in

245   PCO vs. control blastocysts ($P<0.05$) including gap junction proteins and genes involved in p53

246   signaling, calcium signaling, TGF-beta signaling, histidine metabolism, and apoptosis (Table I).

247   Transcriptome analysis of MF blastocysts resulted in some similar gene ontology

248   differences in relation to PCO blastocysts that included: signal transduction, regulation of

249   apoptosis, cell adhesion, reproduction and receptor binding. Unique differences were also

250   observed for MF including: response to stress, regulation of growth, and protein dimerization

251   activity. Pathway analysis of MF vs. control blastocysts revealed enrichment in TGF-beta, ErbB,

252   B cell receptor and GnRH signaling (Table I).

253   Functional annotation of UE blastocysts also had similar outcomes to PCO blastocysts in

254   signal transducer activity, receptor binding, cell differentiation, adhesion and morphogenesis,

255   reproduction, and response to stimulus, among others. Unique differences for UE included:

256   oxidoreductase activity, protein dimerization activity, and monooxygenase activity. Pathway

257   analysis of UE vs. control blastocysts had some similarities (TGF-beta signaling and focal

258   adhesion) compared to the other two groups but many more differences including affected

259   pathways: Type I diabetes, antigen processing, leukocyte migration, autoimmune thyroid

260   disease, systemic lupus erythematosus , mTOR signaling, and adipocytokine signaling (Table I).

261   **Microarray Validation**

262   RT- qPCR was used to validate the microarray data by investigating the expression

263   levels of genes involved in stress response, apoptosis, cell growth and adhesion, and

264   embryonic development. qPCR results confirmed a significantly higher expression of the stress

265   sensing protein activating transcription factor 3 (ATF3) in PCO blastocysts compared to donor

266   controls (P<0.05) and lower levels of anti-apoptotic oocyte-inherited gene (BCL2L10) (P<0.05)

267   and the heat shock proteins HSPA1A and HSPA1B (P<0.05; Figure III). Blastocysts from

268   patients with MF infertility displayed an increased expression of growth differentiating factor 15

269   (GDF15) (P<0.05) and the cell proliferation regulator, INHBA (P<0.05; Figure IV) as observed in

270   the microarray data. Additionally, reduced expression was validated in MF blastocysts for

271   fibroblast growth factor 9 (FGF9) (P<0.05), and left-right determination factors 1 and 2 (LEFTY1,

272   LEFTY2) (P<0.05; Figure IV). Three genes were also confirmed to have reduced expression in

273   blastocysts with UE infertility as observed in the microarray data: Adherens Junctions

274   Associated Protein 1 (AJAP1), cadherin 9 (CDH9), and Laminin Subunit Alpha 4 (LAMA4) (All

275   P<0.05; Figure V).

276

277   **Discussion**

278   This study highlighted that the human blastocyst transcriptome is significantly impacted

279   by the type of patient infertility diagnosis (PCO, MF, and UE). All three of the infertility

280   diagnoses shared transcriptome alterations, with PCO blastocysts displaying the greatest

281   transcriptome variation. An altered blastocyst transcriptome has the potential to impact overall

282   developmental competence, contributing to the infertility observed in patients with these

283   etiologies.

284   The expression of genes involved in stress response and apoptosis were significantly

285   different in PCO blastocysts compared to donor controls, suggesting a PCO environment has a

286   significant impact on the developing blastocyst's transcriptome, including alterations in stress

287   signaling pathways and the regulation of apoptosis. These findings are consistent with those of

288   Wang et al, who reported differential expression of 650 transcripts in the ovaries of women with

289   PCOS compared to normal ovaries and found similar alterations in pathways involved in stress

290   response, apoptosis, and regulation of transcription (Wang et al. , 2014). A higher expression of

291   ATF3 and lower expression of BCL2L10, HSPA1A and HSPA1B in PCO blastocysts was

292   observed in this study compared with donor controls. ATF3, a stress sensor, increases p53

293   protein levels and transcription of p53-responsive genes that result in either cell arrest and DNA

294   repair or apoptosis (Yan et al. , 2005), thereby maintaining DNA integrity. In the developing

295   embryo, highly regulated apoptotic events are critical for embryo homeostasis and survival. The

296   BCL2 proteins are both anti- and pro-apoptotic; BCL2L10 is an anti-apoptotic oocyte-inherited

297   transcript and elimination of BCL2L10 accelerates oocyte death (Guillemin et al. , 2009).

298   HSPA1A and B are involved in embryonic genome activation and decreased expression has

299   been observed in mammalian arrested embryos (Le Masson and Christians, 2011, Pan et al. ,

300   2014). Likewise, gene expression analyses of oocytes from PCOS women also revealed

301   reduced expression in these heat shock proteins (Wood et al. , 2007). Decreased fertilization

302   rates after IVF, as well as a higher risk of miscarriage are associated with the PCO infertility

303   diagnosis. Altered expression levels of each of these genes in PCO may disrupt the normal

304   balance of apoptosis in the pre-implantation embryo, with downstream consequences for

305   implantation and developmental outcomes.

306   Blastocysts derived from MF infertility were significantly altered for TGF-beta and ErbB

307   signaling pathways which are crucial during cell growth and proliferation. GDF15 is a gene

308   belonging to the TGF-beta superfamily and plays a role in regulating inflammatory and apoptotic

309   pathways. The increased expression observed for GDF15 in MF blastocysts is associated with

310   numerous disease states including inflammation and oxidative stress. Likewise, INHBA, which

311   encodes the same TGF-beta superfamily of proteins, was also found to have increased

312   expression in MF blastocysts. It is a negative regulator of gonadal stromal cell proliferation,

313   thus excess expression would lead to inappropriate decreases in cell proliferation which could

314   negatively impact implantation potential. Decreased gene expression in MF blastocysts was

315   observed for FGF9, LEFTY1, and LEFTY2. FGF9 is involved in many biological processes

316   including embryo development, cell growth, and morphogenesis. It has been found to be

317   required for stimulating Erk1/2 activation in differentiating spermatagonia (Tassinari et al. ,

318   2015). LEFTY proteins are critical in sustaining pluripotency and implicated in differentiation of

319   embryonic stem cells (Khalkhali-Ellis et al. , 2016). Inactive LEFTY has been shown to result in

320   embryos that become entirely mesoderm and fail to develop (Hamada et al. , 2002). Poor

321   sperm parameters in MF patients are correlated with fertilization failure and compromised

322   embryo quality and development. Decreases in the expression of these genes could severely

323   impact embryo developmental competence, which is crucial for implantation.

324   Important pathways including cell differentiation and morphogenesis, reproduction, and

325   response to stress were affected from blastocysts derived from patients with UE infertility.

326   These pathways affect embryo growth and development as well as cell adhesion and migration.

327   Decreased expression was observed for three genes involved in cell adhesion and migration:

328   AJAP1, CDH9, and LAMA4. AJAP1 has been observed to be decreased in various cancers and

329   interacts with β-catenin complexes that impact cell cycle function and apoptosis (Zeng et al. ,

330   2014). The decreased expression observed in UE blastocysts could have a negative impact on

331   the balance of apoptosis, possibly leading to inappropriate expression of genes that affect

332   cellular invasion. CDH9 belongs to a family of cell adhesion molecules that regulate

333   morphogenesis and are involved in intracellular signaling pathways (Halbleib and Nelson,

334   2006). These cadherins are responsible for cell-cell adhesion during morula compaction, in

335   addition to playing a role in tissue and organ development (Peyrieras et al. , 1983). Decreased

336 expression would inhibit the ability of both early embryo development, as well as later fetal

337 development in utero. LAMA4 is a laminin that mediates the attachment, migration, and

338 organization of cells into organized tissues during embryonic development. Laminins are vital

339 for organogenesis and have critical functions in several tissues including skin, muscle, and

340 vasculature (Durbeej, 2010). As the etiology of UE infertility is more ambiguous, many adverse

341 outcomes are possible including poor embryo development and IVF failure. The decreased

342 expressed observed in UE blastocysts could have significant consequences to embryo

343 implantation and ongoing development.

344   The similarities between all infertility groups included transcriptome alterations in signal

345 tranducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus.

346 These biological and molecular processes are all inter-related and crucial to embryo

347 development and implantation which are processes characterized by cells that proliferate,

348 migrate, and attach. Receptors are generally transmembrane protein molecules that bind to

349 signaling molecules in response to external stimuli.   Once a receptor protein receives a signal,

350 a series of biochemical reactions is initiated which conveys those signals across a cell,

351 triggering changes in cell function or state, known as signal transduction. An example of this is

352 Hedgehog (Hh) proteins which are expressed during vertebrate development. Hh signaling has

353 been observed during embryonic development and has significance during the growth of

354 reproductive tissues including the gonad and uterus (Walterhouse et al. , 2003). Cellular

355 adhesion, in which cells interact to attach to a surface, regulates signal transduction and is an

356 essential process for embryo implantation into the uterus lining. It is therefore not surprising

357 that all three infertility diagnoses shared blastocysts with transcriptome alterations in these

358 important biological and molecular processes.

359   The differences between each infertility group were more remarkable when studying

360 their pathway analyses. Blastocysts from women with PCO were enriched for apoptosis. This

361 is in concordance with data published showing that ovaries from women with PCO have

362 abnormal apoptotic activity and folliculogenesis (Cai et al. , 2013). On the other hand, signaling

363 pathways from blastocysts with MF infertility were largely involved in cancer processes. Lian et

364 al. also found that infertile men with maturation arrest had hyperactive germ cell proliferation as

365 a result of the inhibition of tumor suppressor IRF1 by its microRNA, miR-383 (Lian et al. , 2010).

366 Interestingly, UE infertility was enriched for pathways involved in mTOR and adipocytokine

367 signaling, both of which are related to various disease states. This could explain some of the

368 difficulties in treating patients with unknown infertility as the cause of their reproductive

369 deficiencies could be the result of anything ranging from environmental to unknown disease risk

370 factors. For example, autoimmune disorders, such as lupus, have been shown to cause a

371 woman's immune system to reject an embryo, thereby preventing implantation into the uterus

372 (Mojarrad et al. , 2013).

373

374 **Conclusions**

375   This novel study suggests that underlying patient infertility diagnosis has an impact on

376 the blastocyst transcriptome, modifying genes that may affect developmental competence and

377 implantation outcomes. Ongoing research determining how transcription alterations are linked to

378 inferior pregnancy outcomes for PCO, MF, and UE patients is crucial to improving IVF success.

379 This is especially true for UE patients as a more defined infertility diagnosis could translate into

380 more targeted clinical management. Understanding how different infertility etiologies contribute

381 to embryo viability may also lead to the development of new laboratory and clinical therapies.

382 An example of this type of clinical advancement is the endometrial receptivity array which

383 identifies endometrial receptivity for patients with repeated implantation failure (Ruiz-Alonso et

384 al. , 2013). Further studies could lead to similar advancements including individualized embryo

385 culture systems and custom stimulation and frozen embryo transfer protocols, thereby

386 improving outcomes for these patients.

387

388 **Acknowledgements**

392

393 **Authors' Roles**

394   BRM performed all microarray and RT-qPCR experiments and analysis and took the

395 lead in preparing the manuscript. JCP collected all blastocyst samples for all experiments.

396 DKG and WBS provided critical review of the manuscript. MKJ designed and oversaw the

397 completion of the study. All authors participated in the editing of the manuscript.

398

399 **Conflict of Interest and Funding**

401

402 **References**

403 Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the

404 globe. Reproductive biology and endocrinology : RB&E 2015;**13**:37.

405 Blakeley P, Fogarty NM, Del Valle I, Wamaitha SE, Hu TX, Elder K, Snell P, Christie L, Robson

406 P, Niakan KK. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq.

407 Development 2015;**142**:3613.

408 Cai L, Ma X, Liu S, Liu J, Wang W, Cui Y, Ding W, Mao Y, Chen H, Huang J et al. Effects of

409 upregulation of Hsp27 expression on oocyte development and maturation derived from

410 polycystic ovary syndrome. PloS one 2013;**8**:e83402.

411 Chaen T, Konno T, Egashira M, Bai R, Nomura N, Nomura S, Hirota Y, Sakurai T, Imakawa K.

412 Estrogen-dependent uterine secretion of osteopontin activates blastocyst adhesion

413 competence. PloS one 2012;**7**:e48933.

414   Daniela Galliano AP. Potential Etiologies of Unexplained Infertility in Females. Unexplained

415   Infertility. 2015. Springer Science, pp. 141-147.

416   Durbeej M. Laminins. Cell Tissue Res 2010;**339**:259-268.

417   Fitzgerald JS, Poehlmann TG, Schleussner E, Markert UR. Trophoblast invasion: the role of

418   intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3).

419   Human reproduction update 2008;**14**:335-344.

420   Fragouli E, Alfarawati S, Spath K, Jaroudi S, Sarasa J, Enciso M, Wells D. The origin and

421   impact of embryonic aneuploidy. Human genetics 2013;**132**:1001-1013.

422   Gardner D, Schoolcraft W. In-vitro culture of human blastocysts. In Jansen R, Mortimer D (eds)

423   Towards Reproductive Certainty: Fertility and Genetics Beyond 1999. 1999. Carnforth,

424   Parthenon Press, pp. 378-388.

425   Guillemin Y, Lalle P, Gillet G, Guerin JF, Hamamah S, Aouacheria A. Oocytes and early

426   embryos selectively express the survival factor BCL2L10. J Mol Med (Berl) 2009;**87**:923-940.

427   Guzick DS, Sullivan MW, Adamson GD, Cedars MI, Falk RJ, Peterson EP, Steinkampf MP.

428   Efficacy of treatment for unexplained infertility. Fertility and sterility 1998;**70**:207-213.

429   Halbleib JM, Nelson WJ. Cadherins in development: cell adhesion, sorting, and tissue

430   morphogenesis. Genes & development 2006;**20**:3199-3214.

431   Hamada H, Meno C, Watanabe D, Saijoh Y. Establishment of vertebrate left-right asymmetry.

432   Nature reviews Genetics 2002;**3**:103-113.

433   Hamatani T, Carter MG, Sharov AA, Ko MS. Dynamics of global gene expression changes

434   during mouse preimplantation development. Developmental cell 2004a;**6**:117-131.

435   Hamatani T, Daikoku T, Wang H, Matsumoto H, Carter MG, Ko MS, Dey SK. Global gene

436   expression analysis identifies molecular pathways distinguishing blastocyst dormancy and

437   activation. Proceedings of the National Academy of Sciences of the United States of America

438   2004b;**101**:10326-10331.

439 Hardy K, Robinson FM, Paraschos T, Wicks R, Franks S, Winston RM. Normal development

440 and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries.

441 Human reproduction 1995;**10**:2125-2135.

442 Janny L, Menezo YJ. Evidence for a strong paternal effect on human preimplantation embryo

443 development and blastocyst formation. Molecular reproduction and development 1994;**38**:36-42.

444 Jones GM, Cram DS, Song B, Kokkali G, Pantos K, Trounson AO. Novel strategy with potential

445 to identify developmentally competent IVF blastocysts. Hum Reprod 2008;**23**:1748-1759.

446 Katz-Jaffe MG, McCallie BR, Janesch A, Filipovits JA, Schoolcraft WB, Gardner DK. Blastocysts

447 from patients with polycystic ovaries exhibit altered transcriptome and secretome. Reproductive

448 biomedicine online 2010;**21**:520-526.

449 Khalkhali-Ellis Z, Galat V, Galat Y, Gilgur A, Seftor EA, Hendrix MJ. Lefty Glycoproteins in

450 Human Embryonic Stem Cells: Extracellular Delivery Route and Posttranslational Modification in

451 Differentiation. Stem Cells Dev 2016.

452 Koivunen R, Laatikainen T, Tomas C, Huhtaniemi I, Tapanainen J, Martikainen H. The

453 prevalence of polycystic ovaries in healthy women. Acta Obstet Gynecol Scand 1999;**78**:137-

454 141.

455 Krishnan A, Muthusami S. Hormonal alterations in PCOS and its influence on bone metabolism.

456 J Endocrinol 2017;**232**:R99-R113.

457 Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos:

458 the Cryotop method. Theriogenology 2007;**67**:73-80.

459 Latham KE, Schultz RM. Embryonic genome activation. Front Biosci 2001;**6**:D748-759.

460 Le Masson F, Christians E. HSFs and regulation of Hsp70.1 (Hspa1b) in oocytes and

461 preimplantation embryos: new insights brought by transgenic and knockout mouse models. Cell

462 stress & chaperones 2011;**16**:275-285.

463   Lian J, Tian H, Liu L, Zhang XS, Li WQ, Deng YM, Yao GD, Yin MM, Sun F. Downregulation of

464   microRNA-383 is associated with male infertility and promotes testicular embryonal carcinoma

465   cell proliferation by targeting IRF1. Cell Death Dis 2010;**1**:e94.

466   Miravet-Valenciano JA, Rincon-Bertolin A, Vilella F, Simon C. Understanding and improving

467   endometrial receptivity. Current opinion in obstetrics & gynecology 2015;**27**:187-192.

468   Mojarrad M, Hassanzadeh-Nazarabadi M, Tafazoli N. Polymorphism of genes and implantation

469   failure. Int J Mol Cell Med 2013;**2**:1-8.

470   Niakan KK, Han J, Pedersen RA, Simon C, Pera RA. Human pre-implantation embryo

471   development. Development 2012;**139**:829-841.

472   Pan X, Kong D, Liu L, Gao F, Zhang X, Tang B, Li Z. Development block of golden hamster

473   ICSI embryos is associated with decreased expression of HDAC1, HSPA1A and MYC. Cell

474   biology international 2014;**38**:1280-1290.

475   Parks JC, McCallie BR, Janesch AM, Schoolcraft WB, Katz-Jaffe MG. Blastocyst gene

476   expression correlates with implantation potential. Fertility and sterility 2011;**95**:1367-1372.

477   Peyrieras N, Hyafil F, Louvard D, Ploegh HL, Jacob F. Uvomorulin: a nonintegral membrane

478   protein of early mouse embryo. Proceedings of the National Academy of Sciences of the United

479   States of America 1983;**80**:6274-6277.

480   Practice Committee of the American Society for Reproductive M. Effectiveness and treatment

481   for unexplained infertility. Fertility and sterility 2006;**86**:S111-114.

482   Quaas A, Dokras A. Diagnosis and treatment of unexplained infertility. Rev Obstet Gynecol

483   2008;**1**:69-76.

484   Ron-el R, Nachum H, Herman A, Golan A, Caspi E, Soffer Y. Delayed fertilization and poor

485   embryonic development associated with impaired semen quality. Fertility and sterility

486   1991;**55**:338-344.

487   Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F,

488   Carrera J, Vilella F, Pellicer A, Simon C. The endometrial receptivity array for diagnosis and

489 personalized embryo transfer as a treatment for patients with repeated implantation failure.

490 Fertility and sterility 2013;**100**:818-824.

491 Sirmans SM, Pate KA. Epidemiology, diagnosis, and management of polycystic ovary

492 syndrome. Clin Epidemiol 2013;**6**:1-13.

493 Tassinari V, Campolo F, Cesarini V, Todaro F, Dolci S, Rossi P. Fgf9 inhibition of meiotic

494 differentiation in spermatogonia is mediated by Erk-dependent activation of Nodal-Smad2/3

495 signaling and is antagonized by Kit Ligand. Cell Death Dis 2015;**6**:e1688.

496 Veeck LL, Bodine R, Clarke RN, Berrios R, Libraro J, Moschini RM, Zaninovic N, Rosenwaks Z.

497 High pregnancy rates can be achieved after freezing and thawing human blastocysts. Fertility

498 and sterility 2004;**82**:1418-1427.

499 Walterhouse DO, Lamm ML, Villavicencio E, Iannaccone PM. Emerging roles for hedgehog-

500 patched-Gli signal transduction in reproduction. Biology of reproduction 2003;**69**:8-14.

501 Wang XX, Wei JZ, Jiao J, Jiang SY, Yu DH, Li D. Genome-wide DNA methylation and gene

502 expression patterns provide insight into polycystic ovary syndrome development. Oncotarget

503 2014;**5**:6603-6610.

504 Wood JR, Dumesic DA, Abbott DH, Strauss JF, 3rd. Molecular abnormalities in oocytes from

505 women with polycystic ovary syndrome revealed by microarray analysis. J Clin Endocrinol

506 Metab 2007;**92**:705-713.

507 Yan C, Lu D, Hai T, Boyd DD. Activating transcription factor 3, a stress sensor, activates p53 by

508 blocking its ubiquitination. The EMBO journal 2005;**24**:2425-2435.

509 Zeng L, Fee BE, Rivas MV, Lin J, Adamson DC. Adherens junctional associated protein-1: a

510 novel 1p36 tumor suppressor candidate in gliomas (Review). International journal of oncology

511 2014;**45**:13-17.

512

513

514

515 **Table I. Significantly altered transcripts and pathways associated with specific infertility**

516 **diagnoses**

517

Infertility Diagnosis	# ↑ Genes (P<0.05; >2-fold)	# ↓ Genes (P<0.05; >2-fold)	Enriched Pathways (P<0.05; >2-fold)
PCO	647	222	p53 signaling, TGF-beta signaling, apoptosis, histidine metabolism
MF	143	205	TGF-beta signaling, ErbB signaling, GnRH signaling, B cell receptor signaling
UE	305	168	mTOR signaling, autoimmune thyroid disease, systemic lupus erythematosus, Type I diabetes, and adipocytokine signaling

518

Figure 1. Differential transcriptome profiles based on infertility diagnosis. Human blastocyst transcriptomes from unexplained infertility (UE) (lanes 1 and 2), polycystic ovaries (PCO) (lanes 3 and 4), male factor (MF) (lanes 5 and 6) and control (lanes 7 and 8). Unsupervised hierarchical clustering clearly separated the transcriptomes of the four groups, with the most significant variation in gene expression observed for the PCO group.
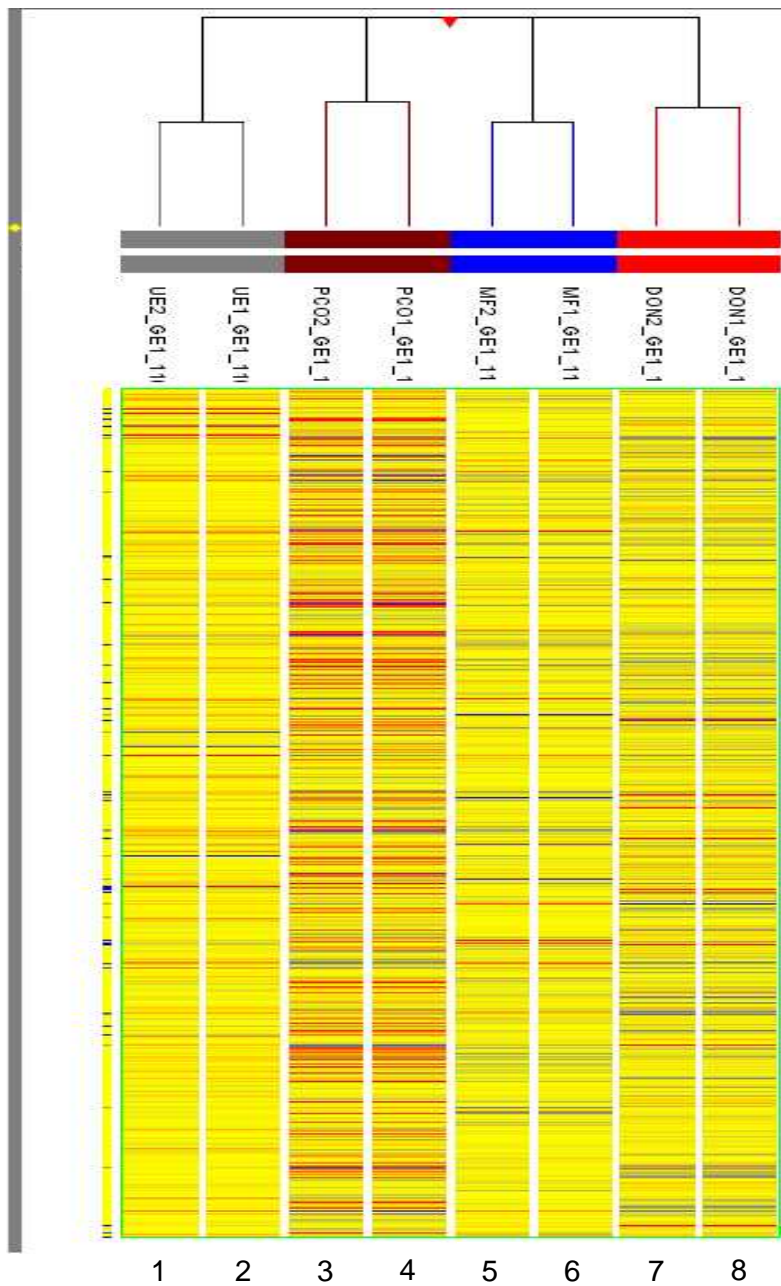
Figure 2. Venn diagram depicting gene overlap of differentially expressed transcripts between the infertility diagnoses groups. P < 0.05; >2-fold; one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction. N = 1385 genes.
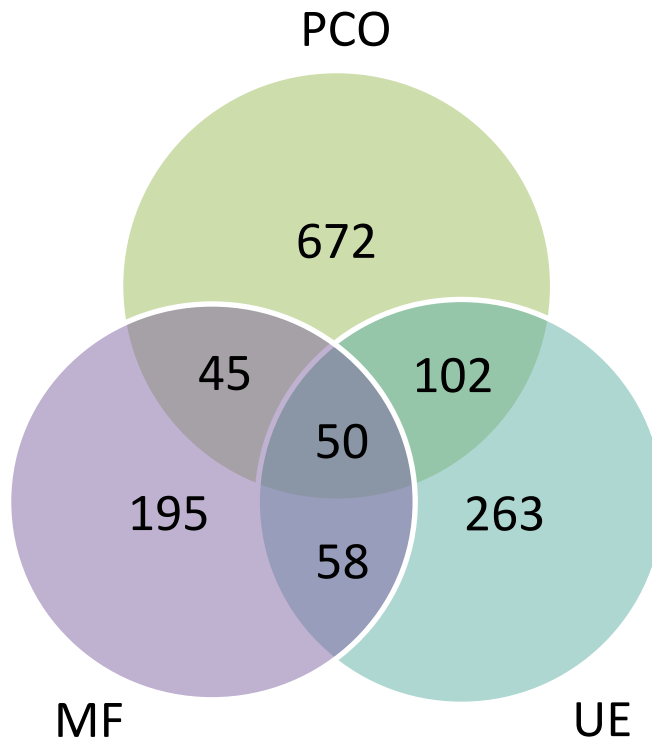
Figure 3. Altered expression of genes involved in apoptosis and stress response in PCO blastocysts. Quantitative PCR (qPCR) was performed to validate expression levels of activating transcription factor 3 (ATF3), BCL2 like 10 (BCL2L10) and heat shock protein family A members 1A and 1B (HSPA1A and HSPA1B) in donor control and PCO blastocysts with peptidylprolyl isomerase A (PPIA) transcription as the constant internal reference gene. A significant increase in ATF3 expression was observed, while BCL2L10, HSPA1A and HSPA1B displayed significantly lower expression in PCO blastocysts, compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test.
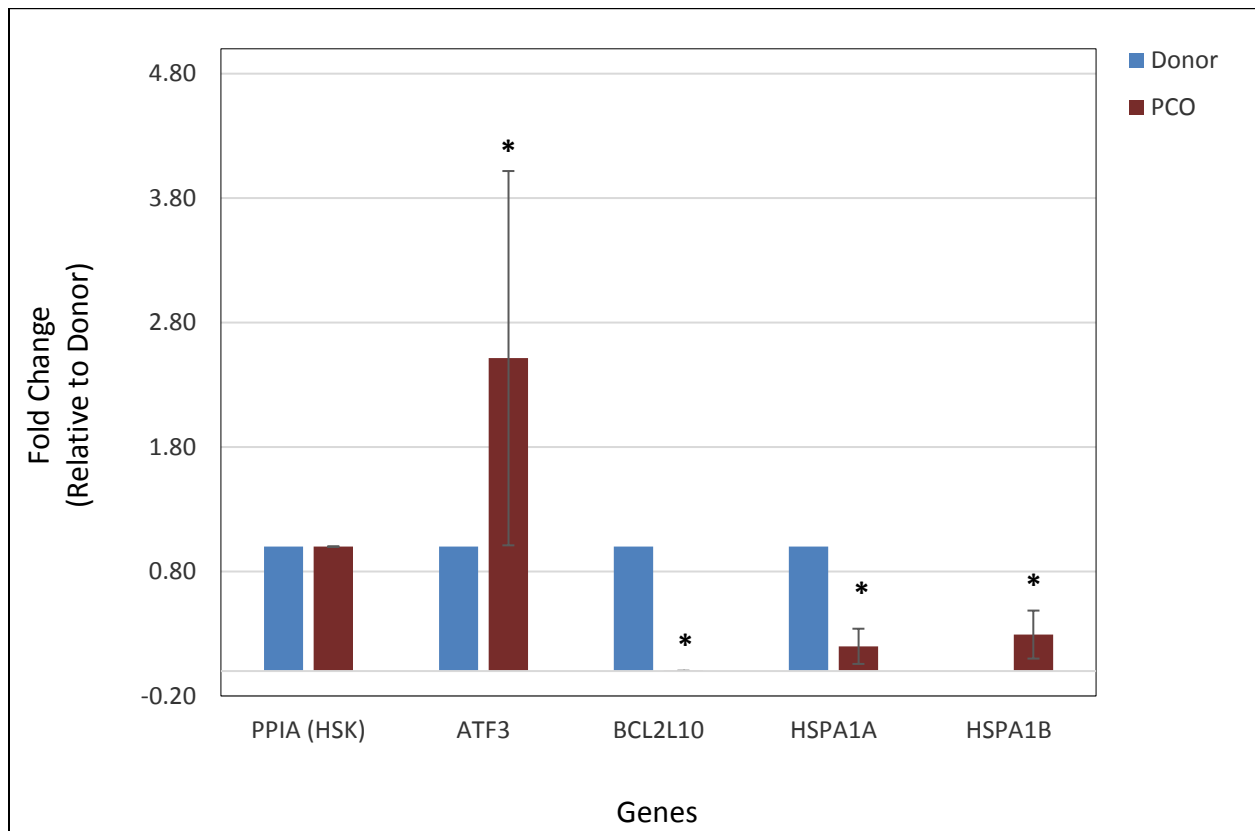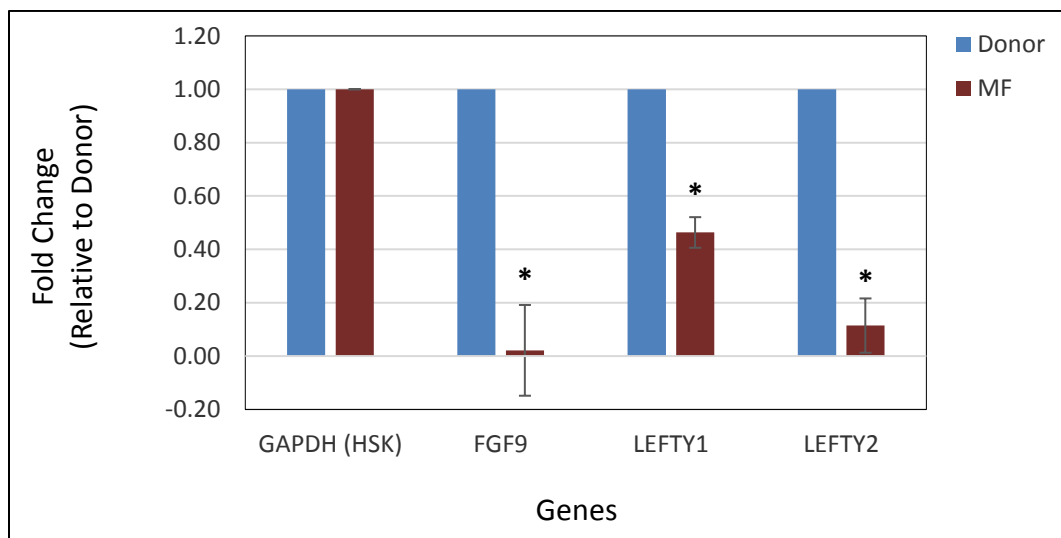
Figure 4. Altered expression of genes involved in cell growth and differentiation in MF blastocysts. qPCR was performed to validate expression levels of growth differentiation factor 15 (GDF15), inhibin beta A subunit (INHBA), fibroblast growth factor 9 (FGF9) and leftright determination factors 1 and 2 (LEFTY1 and LEFTY2) in donor control and MF blastocysts with GAPDH transcription as the constant internal reference gene. (A) Expression of FGF9, LEFTY1 and LEFTY2 was significantly lower and (B) GDF15 and INHBA significantly higher in MF blastocysts compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test.
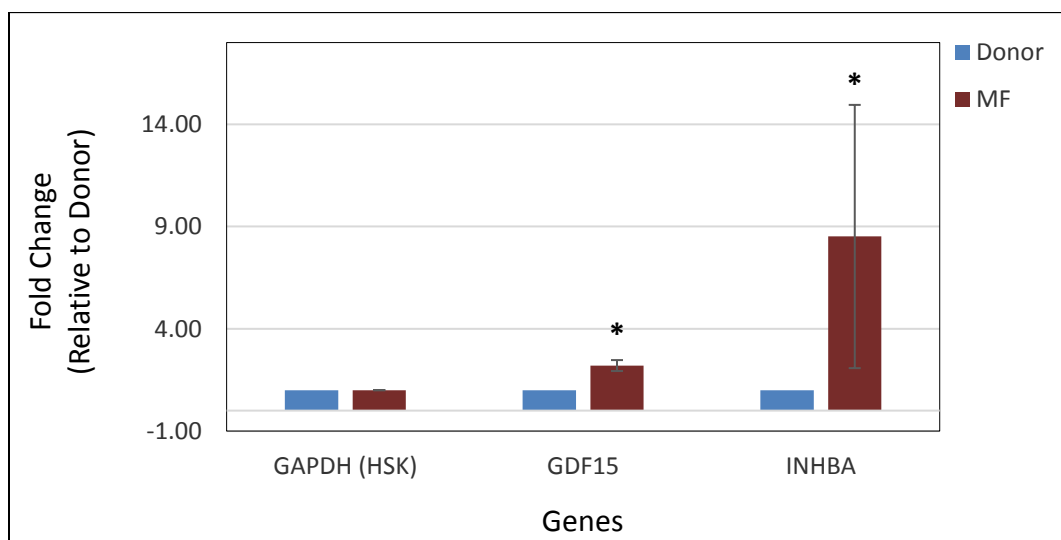
A)

B)

Figure 5. Altered expression of genes involved in cell adhesion and
migration in UE blastocysts. qPCR was performed to validate expression
levels of adherens junctions associated protein 1 (AJAP1), cadherin
9 (CDH9) and laminin subunit alpha 4 (LAMA4) in donor control
and UE blastocysts with GAPDH transcription as the constant internal
reference gene. All three genes were significantly decreased in expression
in UE blastocysts compared to donor controls; *P < 0.05; pairwise
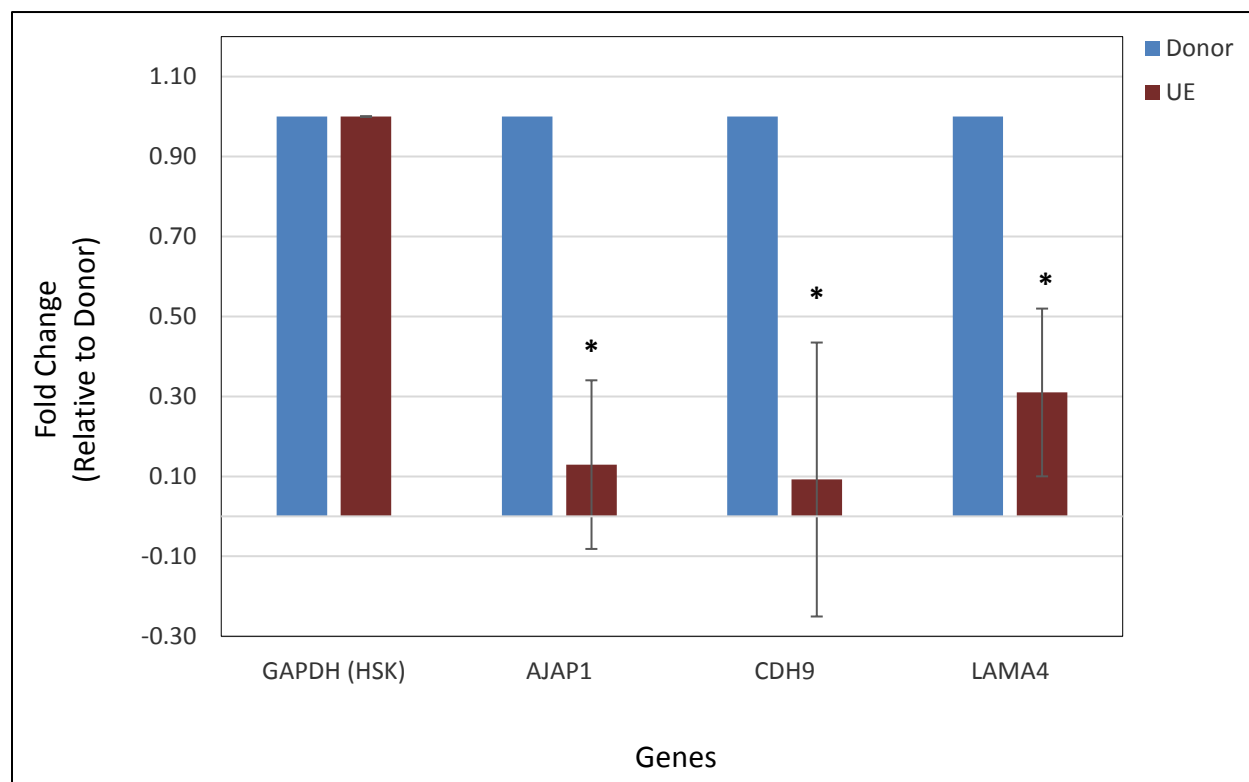fixed reallocation randomization test.

Table 1. Significantly altered transcripts and pathways associated with specific infertility diagnoses

Infertility Diagnosis	# ↑ Genes (P<0.05; >2-fold)	# ↓ Genes (P<0.05; >2-fold)	Enriched Pathways (P<0.05; >2-fold)
PCO	647	222	p53 signaling, TGF-beta signaling, apoptosis, histidine metabolism
MF	143	205	TGF-beta signaling, ErbB signaling, GnRH signaling, B cell receptor signaling
UE	305	168	mTOR signaling, autoimmune thyroid disease, systemic lupus erythematosus, Type I diabetes, and adipocytokine signaling

PCO, polycystic ovaries; MF, male factor infertility; UE, unexplained infertility; TGF, transforming growth factor; ErbB, epidermal growth factor; mTOR, mechanistic target of rapamycin.

Statistical method: one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction.